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# Epstein-Barr Virus Nuclear Proteins EBNA-3A and EBNA-3C Are Essential for B-Lymphocyte Growth Transformation

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**Recombinant Epstein-Barr viruses (EBV) with a translation termination codon mutation inserted into the nuclear protein 3A (EBNA-3A) or 3C (EBNA-3C) open reading frame were generated by second-site homologous recombination. These mutant viruses were used to infect primary B lymphocytes to assess the requirement of EBNA-3A or -3C for growth transformation. The frequency of obtaining transformants infected with a wild-type EBNA-3A recombinant EBV was 10 to 15%. In contrast, the frequency of obtaining transformants infected with a mutant EBNA-3A recombinant EBV was only 1.4% (9 mutants in 627 transformants analyzed). Transformants infected with mutant EBNA-3A recombinant virus could be obtained only by coinfection with another transformation-defective EBV which provided wild-type EBNA-3A in *trans*. Cells infected with mutant EBNA-3A recombinant virus lost the EBNA-3A mutation with expansion of the culture. The decreased frequency of recovery of the EBNA-3A mutation, the requirement for transformation-defective EBV coinfection, and the inability to maintain the EBNA-3A mutation indicate that EBNA-3A is essential or critical for lymphocyte growth transformation and that the EBNA-3A mutation has a partial dominant negative effect. Five transformants infected with mutant EBNA-3C recombinant virus EBV were also identified and expanded. All five also required wild-type EBNA-3C in *trans*. Serial passage of the mutant recombinant virus into primary B lymphocytes resulted in transformants only when wild-type EBNA-3C was provided in *trans* by coinfection with a transformation-defective EBV carrying a wild-type EBNA-3C gene. A secondary recombinant virus in which the mutated EBNA-3C gene was replaced by wild-type EBNA-3C was able to transform B lymphocytes. Thus, EBNA-3C is also essential or critical for primary B-lymphocyte growth transformation.**

Epstein-Barr virus (EBV) is the etiological agent of infectious mononucleosis and is associated with several malignancies, including lymphomas in immunocompromised hosts (9, 70), Burkitt lymphoma (19, 35, 49, 71), nasopharyngeal carcinoma (14, 20, 71), and Hodgkin's disease (4, 62). EBV infects B lymphocytes and certain epithelial cells (27, 33, 59). EBV infection of B lymphocytes causes cell activation (7, 16, 38, 64, 68), continuous cell proliferation, and cell growth transformation as assayed in marmosets or SCID mice (43, 44, 55, 61, 67). Two EBV types, 1 and 2, coexist in most human populations. The two types have distinctive nuclear protein EBNA-LP, -2, -3A, -3B, and -3C genes (1, 13, 54, 56). Type 1 EBV is very efficient at transforming B lymphocytes, while type 2 EBV is very inefficient (52). This type-specific difference in transformation efficiency is primarily due to type-specific differences in the EBNA-2 gene (11, 52). Although EBV can potentially code for 80 to 100 genes (5), only a restricted set of 10 genes are expressed during B-lymphocyte latent infection (reviewed in references 29 and 30). Six genes encode EBNA-LP, -1, -2, -3A, -3B, and -3C. Two genes encode latent infection membrane proteins LMP-1 and LMP-2A/B, and two genes encode nonpolyadenylated small RNAs (EBERs). These genes are presumed to mediate EBV latent infection and growth transformation of B lymphocytes.

Recent advances in EBV recombinant molecular genetics make possible the direct evaluation of the role of the EBNAs, LMPs, or EBERs in EBV-mediated B-lymphocyte growth transformation (10, 11, 17, 34, 36, 63, 66). One approach involves transfecting an EBV-infected cell line (P3HR-1) with cloned EBV DNA fragments and inducing virus replication (11, 17). The transfected DNA can then undergo homologous recombination with the replicating endogenous genome (10, 11, 17, 34, 36, 37, 63, 65, 66). The EBV genome in P3HR-1 cells is competent for lytic infection but is deleted for EBNA-2 and part of EBNA-LP (8, 13, 18, 21, 25, 31, 50), rendering it unable to transform B lymphocytes (39, 42, 51). When P3HR-1 cells are transfected with a cloned wild-type (WT) EBV DNA which includes the DNA deleted from the P3HR-1 EBV genome and replication is induced, the transfected fragment and the P3HR-1 EBV genome recombine with an efficiency of about 1 in $10^{-5}$, resulting in a WT recombinant virus which can transform primary B lymphocytes (11). EBNA-2 proved to be essential for lymphocyte growth transformation, and the deleted part of EBNA-LP was critical (10, 11, 17, 36, 60). A study with the EBERs, which are located 30 kb from EBNA-LP and -2, demonstrated that recombinant EBV carrying an EBER deletion could be made by homologous recombination with a transfected cosmid EBV DNA fragment which included EBNA-LP and -2 (63). The EBER-deleted recombinant virus was still able to transform primary B lymphocytes (63). This phenomenon of second-site recombination was further shown to be independent of physical linkage to the EBNA-LP- and EBNA-2-encoding DNA, and recombinant virus could be made at any site in the EBV genome (65). P3HR-1 EBV genomes which recombined with transfected DNA and were restored for transformation competence also recombined homologously with a second nonlinked transfected DNA fragment with a frequency of 10 to 15% (65, 66). This second-site recombination was used to generate recombinant EBVs in which the P3HR-1 type 2 EBNA-3 genes were replaced with the type 1 EBNA-3 genes (65). Similarly, a

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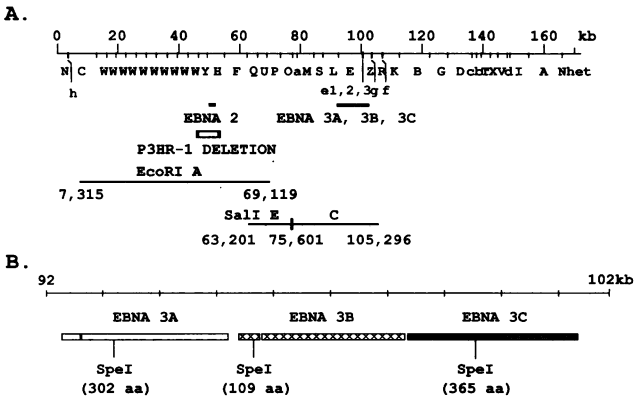


FIG. 1. (A) Schematic representation of the EBV genome indicating the locations of the EBNA-2 and EBNA-3 genes and the P3HR-1 deletion. Also shown are the cloned type 1 EBV *Eco*RI A and *Sal*I E/C cosmid fragments. Prior to transfection, the mutant EBNA-3A and -3C pDVSalI E/C cosmids were digested with *Sal*I to liberate the *Sal*I C fragment, which encodes the EBNA-3 genes. (B) Schematic representation of the *Bam*HI-E region of *Sal*I-C which includes the EBNA-3A, -3B, and -3C genes. Also shown are the fragments amplified by the different PCR primers, the EBNA-3A, -3B, and -3C *Spe*I sites used to terminate translation of the EBNA-3A, -3B, and -3C proteins, and the predicted sizes of the truncated proteins. aa, amino acids.

mutant (MT) EBNA-3B recombinant virus was generated by introducing a stop codon mutation into codon 110 of the 938-codon type 1 EBNA-3B open reading frame (ORF) (66). This EBNA-3B mutation did not affect the ability of the resulting recombinant virus to infect or transform primary B lymphocytes, demonstrating that EBNA-3B is nonessential for primary B-lymphocyte infection or growth transformation (66). In parallel with the EBNA-3B experiments, we assessed the role of EBNA-3A and -3C in B-lymphocyte growth transformation. These latter experiments provided initial indications that EBNA-3A and -3C are important for B-lymphocyte growth transformation. The experiments were extended and further developed, ultimately providing compelling evidence that EBNA-3A and -3C are essential in EBV-mediated primary B-lymphocyte growth transformation.

## MATERIALS AND METHODS

**Plasmid and cosmid construction.** Plasmid pSVNaeI Z (63) was used to induce lytic EBV replication (12). Cosmid *Eco*RI-A (37, 65) was used to obtain recombinant EBV restored for the last two EBNA-LP exons and the EBNA-2 exon. Cosmid *Sal*I-E/C (pDVSECWT) (65) was used to create mutations in EBNA-3A or -3C (Fig. 1). A stop codon was introduced after codon 302 of EBNA-3A or after codon 365 of EBNA-3C in pDVSECWT by partial digestion with *Spe*I. The overhanging *Spe*I ends were filled in with T4 DNA polymerase. A 14-bp *Xba*I linker CTAGTCTAGACTAG with stop codons in all three reading frames was blunt-end ligated into the *Spe*I site and packaged into bacteriophage lambda, using an in vitro packaging extract (Stratagene). Cosmid clones were screened by restriction endonuclease analysis to demonstrate the new *Xba*I site and the absence of other changes in the DNA.

**Cells and cell culture.** The HH514-16 subclone of P3HR-1 (c16) (a gift of George Miller, Yale University) contains a type 2 EBV genome deleted for the EBNA-2 gene and part of the EBNA-LP gene, rendering it nontransforming (24). B95-8 is a marmoset B-cell line immortalized with type 1 EBV (41, 43). BJA-B is an EBV-negative B-lymphoma cell line (40). Human peripheral blood B lymphocytes were obtained from adult seronegative or seropositive donors. T cells were removed with 2-aminoethyl isothiouronium bromide (Sigma)-treated sheep erythrocytes. Cell lines were maintained in complete medium, which consisted of RPMI 1640 medium supplemented with 10% inactivated fetal bovine serum, glutamine, and 10 μg of gentamicin per ml.

**Cosmid transfection.** P3HR-1 c16 cells were transfected with cloned type 1 EBV *Eco*RI A and wild-type or mutant *Sal*I C DNA fragments after release of the *Sal*I C and *Sal*I E fragments from the EBV pDVSalI E/C cosmid vector DNA by *Sal*I digestion. Ten micrograms of cosmid *Eco*RI-A DNA and 50 μg of WT or MT cosmid *Sal*I-E/C DNA were mixed with 25 μg of pSVNaeI Z and used to electroporate $10^7$ P3HR-1 c16 cells. For electroporation, the cells were harvested during log-phase growth, washed once with complete medium, and resuspended in 400 μl of complete medium with DNA in a cuvette (0.4-cm gap; Bio-Rad). Following a 10-min incubation at 25°C, the culture was pulsed with 200 V at 960 μF. Cells were immediately diluted into 20 ml of complete medium. Lytic EBV infection was activated in B-lymphoblastoid cell lines (LCLs) by transfection with 25 μg of pSVNaeI Z under similar conditions.

**Primary B-lymphocyte infections.** Primary human B lymphocytes were infected with filtered (0.45-μm-pore-size filter) culture supernatant obtained from P3HR-1 c16 cells 3 days after transfection. Intracellular virus was first released into the medium by three cycles of freezing and thawing. For some initial experiments, virus was concentrated by centrifugation at 8,800 × *g* for 2 h before filtration. Virus was incubated for 2 h at 37°C with $10^7$ T-cell-depleted human peripheral blood mononuclear cells. Infected cells were resuspended in complete medium at a concentration of $3.3 \times 10^5$ cells per ml, and 150 μl ($5 \times 10^4$ cells) was put into each well of a 96-microwell plate. The cultures were fed at 14 days postplating with 100 μl of complete medium. LCLs were macroscopically visible 3 to 5 weeks after plating.

Lytic infection was induced in infected primary B lymphocytes by transfection with pSVNaeI Z and tetradecanoylphorbol acetate treatment. The resultant virus was passed through a 0.22-μm-pore-size filter and used to infect T-cell-depleted peripheral blood lymphocytes. For P3HR-1 complementation, P3HR-1 cells were reactivated by transfection with 25 μg of pSVNaeI and cultured for 5 to 6 days. Culture supernatant was then harvested, filtered through a 0.22-μm-pore-size filter, and mixed at 1/2, 1/10, and 1/40 with different dilutions of recombinant virus from infected LCLs. The mixed supernatants were then used to infect primary B lymphocytes. Cells were plated at $5 \times 10^4$ cells per well in a total volume of 150 μl. The cultures were fed at 14 days after plating with 100 μl of complete medium. LCL outgrowth was visible 3 to 5 weeks after plating. As irradiation controls, $5 \times 10^4$ irradiated LCLs were plated separately.

**Polymerase chain reaction (PCR) and DNA sequence analyses. (i) PCR primers.** Oligonucleotide primers for amplification of distinctive fragments from type 1 versus type 2 EBNA-3A, -3B, or -3C (56) or from MT versus WT EBNA-3A or -3C were synthesized on an Applied Biosystem model 391 oligonucleotide synthesizer. The EBNA-3A *Spe*I-specific primers TCAGCGTGTTACATTTATGG (sense) and TAAGCAAGTCTATCCCATAC (antisense) correspond to bases 93143 to 93162 and 93339 to 93320, respec-

tively (5). These primers flank the EBNA-3A *Spe*I site at 93234 and amplify a 197-bp fragment from WT EBNA-3A and a 215-bp fragment from MT EBNA-3A DNA as a result of blunt-end insertion of the 14-bp *Xba*I/stop codon linker. The EBNA-3C *Spe*I-specific primers GTAATCCAGAACG CATTTCG (sense) and ACATGACGGGCCTCTGCTGT (antisense) correspond to bases 99456 to 99,475 and 99663 to 99644. These primers flank the EBNA-3C *Spe*I site at 99537 and amplify a 208-bp fragment from WT type 1 EBV DNA (the EBNA-3C *Spe*I primers are specific for type 1 EBV and will not amplify type 2 EBV) and a 226-bp fragment from MT type 1 EBNA-3C DNA containing the *Xba*I linker. Primers (P3HR-1-Del) which flank the P3HR-1 deletion were synthesized and amplify a 257-bp fragment from DNA containing the deletion and do not amplify from DNA restored for the deletion. The P3HR-1-Del primers TCCTCGTCCAGCAAG AAGAG (sense) and TAGCCCTGTGCCATGGGGAC (antisense) correspond to bases 45507 to 45,526 and 52570 to 52551.

**PCR and DNA sequencing.** DNA was prepared from $0.5 \times 10^5$ to $1 \times 10^5$ cells harvested from microwell cultures. Cells were resuspended in 0.2× phosphate-buffered saline, boiled for 10 min, and digested with 1/10 volume of proteinase K (10 mg/ml; Sigma) for 30 min at 55°C. Proteinase K was inactivated by incubation at 95°C for 20 min. DNA was amplified in a Perkin-Elmer thermal cycler, using 10 to 20 μl of DNA in a 50-μl reaction. PCR-amplified DNA was analyzed by electrophoresis in 1% ME–2% NuSieve agarose (FMC) gels and visualized by staining with ethidium bromide. PCR products were sequenced by the dideoxy method (57).

**Immunoblot and Southern blot analyses.** Expression of latent EBV proteins was evaluated by electrophoresis of denatured infected cell proteins in 7.0% denaturing polyacrylamide gels and immunoblotting with human EBV immune serum. For Southern blots, 15 μg of cell DNA was digested with restriction enzyme *Bcl*I (MT EBNA-3A hybridizations) or *Sal*I and *Xba*I (MT EBNA-3C hybridizations) in 200 μl. DNA was then precipitated, dried briefly in a vacuum dryer, and resuspended in 40 μl of loading buffer. The DNA fragments were size fractionated by electrophoresis through a 0.8% agarose gel. DNA was then transferred to an activated nylon membrane (GeneScreen Plus; New England Nuclear). The filters were hybridized with a $^{32}$P-labeled EBNA-3A (MT EBNA-3A hybridizations) or EBNA-3C (MT EBNA-3C hybridizations) cDNA probe.

## RESULTS

**General strategy for mutating the EBNA-3A and -3C genes.** A 14-bp *Xba*I site oligonucleotide linker with amber codons in all frames was inserted into the *Spe*I site after codon 365 for EBNA-3C, codon 302 for EBNA-3A, or codon 109 for an EBNA-3B control (Fig. 1). Two independently derived and validated recombinant cosmid DNAs for the EBNA-3A or -3C mutation were used in approximately equal numbers of experiments to minimize potential cloning artifacts. Transfection with the cloned EBV fragments and induction of lytic infection resulted in homologous recombination between the transfected fragments and the endogenous P3HR-1 viral DNA. Recombination with the *Eco*RI A fragment restored the deleted EBNA-LP and EBNA-2 genes, relieving their requirement for transformation and thereby permitting the selection for P3HR-1 genomes which had undergone homologous recombination. Recombinant virus was used to infect primary B lymphocytes, which were then plated in 96-microwell plates, and the resultant transformed LCLs were analyzed by PCR to identify EBNA-3 recombinant virus and to assess parental P3HR-1 coinfection.

**Derivation of MT EBNA-3C recombinant EBV.** Transfection of P3HR-1 with MT EBNA-3C *Sal*I-C cosmid DNA resulted in the recovery of five LCLs infected with MT EBNA-3C virus. The frequency of recovery an MT EBNA-3C recombinant virus was 2%, while in experiments done in parallel with the WT EBNA-3C *Sal*I-C cosmid, the frequency of recovering WT type 1 EBNA-3C recombinant virus was 3% (65). The low frequency of obtaining WT EBNA-3C recombinant virus compared with the 10 to 15% observed for obtaining WT EBNA-3A and -3B recombinant virus (65) was probably due to the EBNA-3C gene being near the end of the *Sal*I-C cosmid DNA (Fig. 1).

**Analysis of the EBV genomes in cell lines coinfected with MT EBNA-3C recombinant EBV and parental P3HR-1 EBV.** Because of the low WT EBNA-3C recombination frequency, definitive evidence for a decrease in frequency with an MT EBNA-3C would require too many experiments to be feasible. We therefore focused on the five LCLs which had MT EBNA-3C recombinant EBV genomes. Four of the five LCLs maintained the MT EBNA-3C genome on expansion to $2 \times 10^6$ cells; the fifth LCL lost the MT EBNA-3C genome, presumably as a result of secondary recombination with a coinfecting P3HR-1 genome. PCR analysis with type-specific EBNA-3C primers demonstrated the presence of both type 1 and 2 EBNA-3C genes in each of the four MT EBNA-3C virus-infected LCLs (Fig. 2A, lanes 5 to 8). The presence of the type 1 EBNA-3C gene was confirmed by PCR with EBNA-3C *Spe*I site-specific primers, which are specific for type 1 EBNA-3C and amplify across the mutated EBNA-3C *Spe*I restriction site. Amplification of DNA from the MT EBNA-3C virus-infected LCLs yielded a 226-bp fragment (Fig. 2B, lanes 5 to 8), identical in size to the fragment amplified from the MT EBNA-3C control cosmid DNA (Fig. 2B, lane 4), which was larger than the 208-bp amplified from the WT EBNA-3C control cosmid DNA (Fig. 2B, lane 3). The stop codon mutation was further confirmed by *Xba*I digestion. Two smaller fragments for the MT EBNA-3C cosmid control PCR-amplified fragment (Fig. 2C, lane 4) and for each of the MT EBNA-3C virus-infected LCL PCR-amplified fragments (Fig. 2C, lanes 5 to 8) were generated following *Xba*I digestion. The WT EBNA-3C cosmid control PCR-amplified fragment was not digested by *Xba*I (Fig. 2C, lane 3). The presence of the mutation was also confirmed by sequencing the PCR-amplified fragments (data not shown).

The presence of both type 1 and 2 EBNA-3C genes (Fig. 2) indicated the MT EBNA-3C virus-infected LCLs were likely coinfected with MT EBNA-3C virus and parental P3HR-1 virus. Amplification of DNA from MT EBNA-3C virus-infected LCLs and control cell lines with primers (P3HR-1-Del) which flank the P3HR-1 deletion resulted in amplification of a 256-bp fragment from E3CT-43, E3CT-80, and E3CT-105 DNA (Fig. 3A, lanes 5 to 7), identical in size to the fragment amplified from parental P3HR-1 DNA (Fig. 3A, lane 3), confirming the presence of parental P3HR-1 virus in these three MT EBNA-3C virus-infected LCLs. Parental P3HR-1 EBV DNA was not detected in E3CT-119 DNA (Fig. 3A, lane 8), indicating that nonhomologous or partially homologous recombination might be the reason why E3CT-119 contains both MT type 1 and WT type 2 EBNA-3C DNA. B95-8 DNA does not contain a deletion in this region, and as expected, no 256-bp fragment was amplified (Fig. 3A, lane 4). Two smaller bands were present, and these are likely to be due nonspecific amplification. Southern hybridization

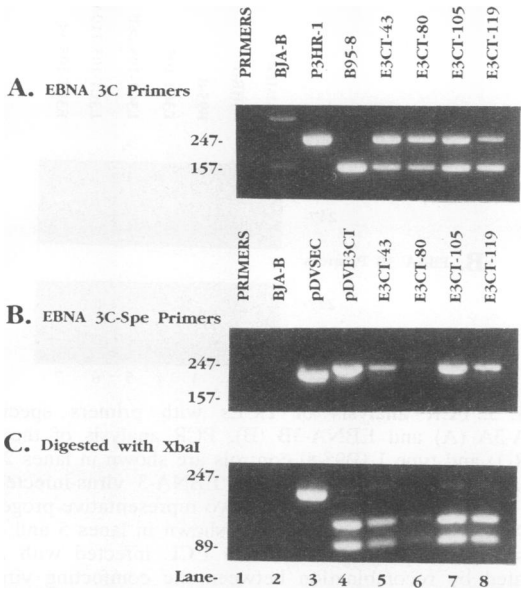


FIG. 2. PCR analyses of LCLs infected with MT EBNA-3C recombinant EBV. (A) PCR analyses with EBNA-3C primers amplify a 153-bp fragment from type 1 and a 246-bp fragment from type 2 EBV DNA. (B) PCR analyses with EBNA-3C *Spe*I site-specific primers amplify a 208-bp fragment from DNA with a wild-type EBNA-3C *Spe*I site or a 226-bp fragment from DNA with a mutated EBNA-3C *Spe*I site, which has the *Xba*I/stop codon oligonucleotide. (C) The PCR products shown in panel B were digested with *Xba*I to confirm the new *Xba*I site in the mutant DNA. Lanes: 1 and 2, control amplifications with no added DNA and DNA from a EBV-negative B-lymphoma cell, respectively; 3 and 4, control amplifications with type 2 P3HR-1 EBV DNA and type 1 B95-8 DNA, respectively (A), and wild-type pDVSEC and mutant EBNA-3C pDVE3CT cosmid DNA, respectively (B and C); 5 to 8, amplification with DNA from four MT EBNA-3C virus-infected LCLs. The primers are indicated in Fig. 1. Sizes (base pairs) of *Rsa*I fragments of ϕX174 DNA are shown on the left.

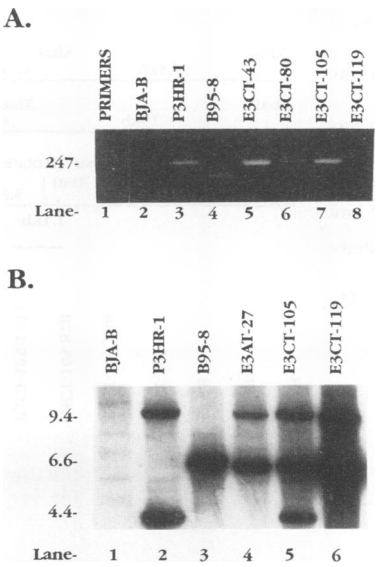


FIG. 3. Evidence that LCLs infected with MT EBNA-3C recombinant virus are also coinfected with parental P3HR-1 virus. (A) PCR analyses with primers which flank the EBNA-LP and EBNA-2 P3HR-1 deletion (P3HR-1-Del) amplify a 256-bp fragment from P3HR-1 (lane 3). The corresponding fragment from nondeleted WT B95-8 DNA (lane 4) does not amplify because of the separation of the primers by more than 6 kb of DNA. The PCR fragments amplified from B95-8 DNA are smaller than 256 bp and probably result from nonspecific amplification. Lane 1 and 2 are negative control amplifications with no added DNA and DNA from an EBV-negative BL cell, respectively. Amplifications of DNA from LCLs infected with MT EBNA-3C recombinant EBV are shown in lanes 5 to 8. The positions and sizes (base pairs) of *Rsa*I fragments of ϕX174 are shown on the left. (B) Southern hybridization of DNA digested with *Bam*HI and probed with a radiolabeled EBV *Bam*HI H fragment. The *Bam*HI H fragment of P3HR-1 is smaller (4.1 kb; lane 2) than that of B95-8 (6.0 kb; lane 3) because of the deletion of DNA including the last exons of EBNA-LP and the EBNA-2 exon. Hybridizations of DNA from LCLs infected with MT EBNA-3A EBV recombinant (E3AT) or with mutant EBNA-3C EBV recombinant (E3CT) virus are in lanes 3 to 5. The EBV *Bam*HI H fragment also hybridized to the 9.7-kb *Bam*HI B1 fragment. Size markers (kilobase pairs) are shown on the left.

with a *Bam*HI-H probe confirmed the PCR results. Hybridization of a radiolabeled *Bam*HI-H probe to *Bam*HI-digested E3CT-105 DNA (Fig. 3B, lane 5) detected the presence of both the recombinant B95-8-type 6.0-kb *Bam*HI H fragment (Fig. 3B, lane 3) and the parental P3HR-1-type 4.1-kb *Bam*HI H fragment (Fig. 3B, lane 2). E3CT-119 DNA had only the B95-8-type 6.0-kb *Bam*HI H fragment (Fig. 3B, lane 6). These results demonstrate that the MT EBNA-3C virus-infected LCLs E3CT-43, E3CT-80, and E3CT-105 were coinfected with a genome restored for EBNA-LP/2 and a P3HR-1-deleted EBNA-LP/2 genome.

**Loss of mutant EBNA-3C recombinant virus with long-term passage of coinfected LCLs.** Expansion of the initial five MT EBNA-3C virus-infected LCLs resulted in loss of the MT EBNA-3C genome in three of the LCLs (including the one which lost the EBNA-3C mutation on expansion beyond $2 \times 10^5$ cells) following 1 to 3 months of passage in culture. Slow loss of the MT EBNA-3C genome is probably due to a low level of spontaneous virus replication and secondary recombination between the MT EBNA-3C and parental P3HR-1 EBV genomes. P3HR-1, which is deleted for EBNA-LP/2 and WT for type 2 EBNA-3C, can recombine with the MT EBNA-3C genome, which is restored for WT EBNA-LP/2 and mutant in type 1 EBNA-3C, resulting in a WT EBNA-LP/2 genome with the WT type 2 EBNA-3C gene from P3HR-1. The newly recombined genome containing WT EBNA-LP/2 and WT type 2 EBNA-3C would be fully transforming, resulting in gradual loss of the presumed nontransforming MT EBNA-3C genome. One coinfected MT EBNA-3C virus-infected LCL (E3CT-105) maintained both the P3HR-1 and MT EBNA-3C genomes and was further analyzed.

**Detailed characterization of EBV genomes in a cell line which maintained MT EBNA-3C recombinant and parental P3HR-1 genomes in a balanced state.** Southern hybridization with an EBNA-3C-specific probe confirmed that the MT EBNA-3C recombinant genome in E3CT-105 had the MT type 1 EBNA-3C DNA in place of the WT type 2 EBNA-3C DNA. DNA from E3CT-105 LCL and control LCLs were digested with *Xba*I and *Sal*I and probed with radiolabeled EBNA-3C cDNA. Figure 4A shows a schematic representation of the *Xba*I and *Sal*I restriction sites around EBNA-3C in WT type 1, WT type 2, and MT type 1 EBNA-3C DNA. Type 1 EBV has a single *Xba*I site at 99398 in the EBNA-3C ORF (98,389-101,788). Hybridization of a Southern blot of *Xba*I- and *Sal*I-digested B95-8 type 1 EBV DNA with an

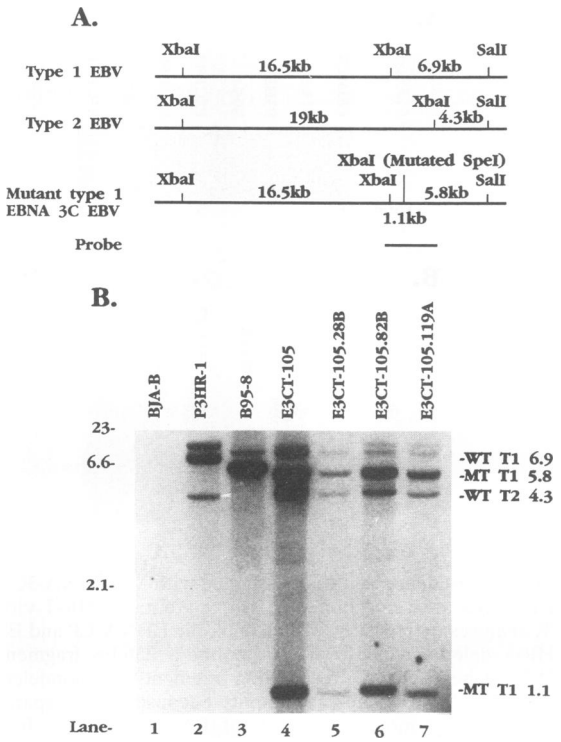


FIG. 4. Southern blot of the EBNA-3C DNA in LCLs infected with MT EBNA-3C recombinant virus, which is consistent with homologous recombination. DNA was digested with *Sal*I and *Xba*I and probed with a labeled EBNA-3C cDNA. (A) Schematic representation of the WT type 1, WT type 2, and MT type 1 EBNA-3C DNAs, indicating the EBNA 3C *Spe*I site and the expected *Sal*I-*Xba*I fragments from each DNA. Type 2 EBV DNA has an *Xba*I site at 100944, while type 1 EBV DNA lacks the that site and has a distinctive site at 98398. MT type 1 EBNA-3C DNA has a new *Xba*I site at 99537, created by insertion of the linker into the EBNA-3C *Spe*I site. The WT type 1 6.9-kb *Xba*I-*Sal*I fragment is cut by *Xba*I at 99537 to give 5.8- and 1.1-kb fragments. (B) Southern blot of *Sal*I- and *Xba*I-digested LCL DNA hybridized with labeled an EBNA-3C cDNA probe. A digest of EBV-negative BJA-B DNA is shown in lane 1. Digests of P3HR-1 and B95-8 DNA are shown in lanes 2 and 3. The 10-kb fragment seen in the P3HR-1 digest is likely due to incomplete digestion at the 105296 *Sal*I site, resulting in a 10-kb *Xba*I fragment (100944 to 110760). A digest of E3CT-105 LCL DNA infected with a MT EBNA-3C recombinant EBV is shown in lane 4. Progeny LCLs infected with virus from E3CT-105 are shown in lanes 5 to 7. Size markers (kilobase pairs) are shown on the left.

EBNA-3C probe resulted in a prominent 6.9-kb fragment (Fig. 4B, lane 3). In contrast, in type 2 EBV DNA, the *Xba*I site is not present and another *Xba*I site is present about 2.5 kb 3′ to the type 1 site at kb 100944. Hybridization of the an EBNA-3C cDNA to P3HR-1 type 2 DNA resulted in 4.3-kb fragment instead of the type 1 6.9-kb fragment (Fig. 4B, lane 2). MT type 1 EBNA-3C DNA has an additional *Xba*I site inserted 99537 so that the type 1 6.9-kb fragment is cleaved into 1.1- and 5.8-kb fragments. E3CT-105 DNA digested with *Xba*I and *Sal*I yielded the expected 1.1- and 5.8-kb fragments, demonstrating the presence of the stop codon/*Xba*I mutation in the type 1 EBNA-3C gene. E3CT-105 also yielded the expected type 2 4.3-kb fragment, indicating the presence of the type 2 EBNA-3C gene (Fig. 4B, lane 4). These results demonstrate that E3CT-105 contains both a WT type 2 EBNA-3C gene and an MT type 1 EBNA-3C

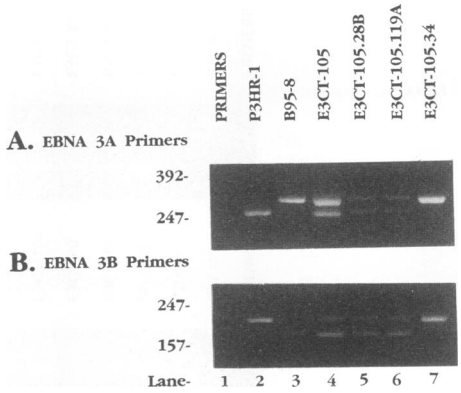


FIG. 5. PCR analysis of LCLs with primers specific for EBNA-3A (A) and EBNA-3B (B). PCR analysis of the type 2 (P3HR-1) and type 1 (B95-8) controls are shown in lanes 2 and 3. PCR analysis of the parental MT EBNA-3 virus-infected LCL E3CT-105 is shown in lane 4, and two representative progeny MT EBNA-3C virus-infected LCLs are shown in lanes 5 and 6. PCR analysis of the progeny CT-105.34 LCL infected with a virus generated by recombination between the coinfecting viruses in E3CT-105 is shown in lane 7. Size markers (base pairs) of *Rsa*I fragments of ϕX174 are shown on the left.

gene. These results taken together with *Bam*HI Southern hybridization results indicate that E3CT-105 is coinfected with deleted EBNA-LP/2 and WT type 2 parental P3HR-1 virus and WT EBNA-LP/2 and MT type 1 EBNA-3C recombinant virus.

PCR analysis of E3CT-105 with the EBNA-3A and EBNA-3B primers, which distinguish between type 1 and type 2 DNA (Fig. 1), demonstrated that the MT EBNA-3C genome in E3CT-105 has type 1 EBNA-3A (Fig. 5A, lane 4) and EBNA 3B (Fig. 5B, lane 4). Thus, the recombination between the transfected *Sal*I-C DNA and P3HR-1 EBV DNA extends from 5′ of the EBNA-3A primers to 3′ of the EBNA-3C primers.

Immunoblot analysis of E3CT-105 with type 1 EBNA-3C- and EBNA-3B-specific-antibodies confirmed the presence of type 1 EBNA-3B and the absence of type 1 EBNA-3C protein expression in E3CT-105 (45, 47). Figure 6 shows that duplicate immunoblots of proteins from E3CT-105 (lane 3),

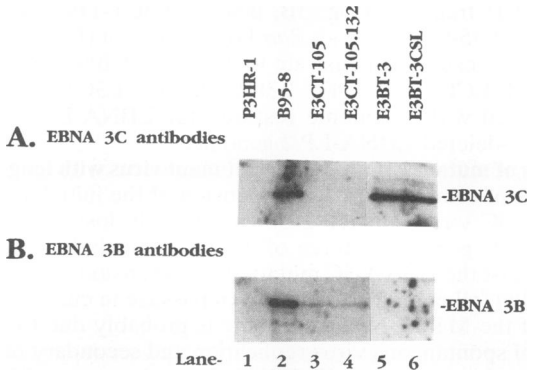


FIG. 6. Failure of immunoblot analysis to detect EBNA-3C protein expression in MT EBNA-3C virus-infected LCLs. Duplicate immunoblots were incubated with affinity-purified type 1-specific EBNA-3C (A) or EBNA-3B (B) antibodies (45, 47). E3BT-3 and E3BT-3CSL are MT EBNA-3B LCLs (66) which express type 1 EBNA-3C but not type 1 EBNA-3B.

type 2 P3HR-1 (lane 1), type 1 B95-8 (lane 2), and two WT type 1 EBNA-3C-expressing recombinant LCLs (E3BT-3 [lane 5] and E3BT-3CSL [lane 6]) reacted with type 1 EBNA-3C-specific (Fig. 6A) or EBNA-3B-specific (Fig. 6B) antibodies. Type 1 EBNA-3B was detected in E3CT-105 (Fig. 6B, lane 3) and in the B95-8 type 1 control (Fig. 6B, lane 2); neither E3BT-3 nor E3BT-3CSL expresses EBNA 3B as a result of a mutation inserted into the EBNA-3B ORF (66). Type 1 EBNA-3C was detected in B95-8 (Fig. 6A, lane 2), E3BT-3 (Fig. 6A, lane 5), and E3BT-3CSL (Fig. 6A, lane 6) but not in E3CT-105, demonstrating the effectiveness of the linker insertion mutation in terminating the translation of EBNA-3C.

**MT EBNA-3C recombinant EBV can initiate growth transformation of primary B lymphocytes only when WT EBNA-3C is provided in *trans*.** To verify the inability of the MT EBNA-3C recombinant virus to initiate growth transformation, lytic infection was activated in E3CT-105, and the filtered (0.22-μm-pore-size filter) virus was used to infect primary B lymphocytes. If EBNA-3C is essential for lymphocyte growth transformation, then the MT EBNA-3C virus should transform only primary B lymphocytes as a result of coinfection with P3HR-1 virus. Addition of exogenous parental P3HR-1 virus would be expected to increase the number of LCLs transformed by MT EBNA-3C genome. LCLs could also arise as a result of infection with a virus resulting from secondary recombination between the MT EBNA-3C and parental P3HR-1 EBV genomes in the lytically infected E3CT-105 (see above). The results of two reactivation experiments are shown in Table 1 and Fig. 7 and 8. Of the 72 LCLs established in the absence of exogenous WT P3HR-1 virus, none were infected with the MT EBNA-3C virus. All 72 LCLs had only WT type 2 EBNA-3C, presumably as a consequence of recombination between the two genomes in E3CT-105. These results contrast strikingly with the ease of obtaining WT type 1 EBNA-3A and -3B recombinant virus in LCLs after activating lytic infection in an LCL (T1E3-107) coinfected with WT EBNA-LP/2 and WT type 1 EBNA-3A and -3B double recombinant (WT EBNA-3A and -3B) virus (65) and parental P3HR-1 virus. Reactivation of lytic infection in T1E3-107 and serial passage through primary B lymphocytes resulted in 16 of 37 LCLs being singly infected with the WT type 1 recombinant EBNA-3A and -3B virus (data not shown). Thus, the failure to obtain LCLs infected with the MT EBNA-3C virus alone is evidence that MT EBNA-3C recombinant EBV is unable to transform B lymphocytes alone and that EBNA-3C is essential for B-lymphocyte transformation.

As expected, the MT EBNA-3C virus could transform primary B lymphocytes when cells were simultaneously infected with P3HR-1, which provided WT type 2 EBNA-3C in *trans*. When B lymphocytes were infected with a 1/2 or 1/10 dilution of virus from E3CT-105 and a 1/2 dilution of parental P3HR-1 EBV, 57 of the 150 resultant LCLs (38%) were coinfected with MT EBNA-3C and P3HR-1 virus. The remaining progeny LCLs were infected with WT type 2 EBNA-3C secondary recombinant virus produced during lytic infection in E3CT-105. With a higher dilution of exogenous P3HR-1 virus, fewer LCLs infected with the MT EBNA-3C virus were obtained. With 1/10 and 1/40 dilutions of P3HR-1, the frequencies of recovering the MT EBNA-3C virus in the progeny LCLs decreased to 23% (31 of 135) and 0% (0 of 68), respectively. Again, all of the LCLs infected with MT EBNA-3C virus were coinfected with parental P3HR-1 virus. The recovery of MT EBNA-3C virus only in LCLs with parental P3HR-1 virus coinfection indicates the stringency of the requirement for WT EBNA-3C in primary B-lymphocyte growth transformation. These data also confirm the linkage of the double recombinant WT EBNA-LP/2 and MT EBNA-3C genome in E3CT-105.

TABLE 1. Recovery of MT EBNA-3C virus by complementation with P3HR-1 virus

| Culture[a] | No. of positive LCLs/total no. analyzed | | |
|---|---|---|---|
| | P3-Del primers[b] | E3C-*Spe*I primers[c] | E3C primers[d] |
| Expt 1 | | | |
| 1/2D CT-105 | 0/10 | 0/10 | 0/10 |
| 1/2D CT-105–1/2D P3HR-1 | 19/37 | 10/37 | 10/37 |
| 1/2D CT-105–1/10D P3HR-1 | 3/29 | 3/29 | 3/29 |
| 1/2D CT-105–1/40D P3HR-1 | 0/8 | 0/8 | 0/8 |
| 1/10D CT-105 | 0/17 | 0/17 | 0/17 |
| 1/10D CT-105–1/2D P3HR-1 | 14/19 | 7/19 | 7/19 |
| 1/10D CT-105–1/10D P3HR-1 | 8/17 | 7/17 | 7/17 |
| 1/10D CT-105–1/40D P3HR-1 | 0/16 | 0/16 | 0/16 |
| Expt 2 | | | |
| 1/2D CT-105 | 0/33 | 0/33 | 0/33 |
| 1/2D CT-105–1/2D P3HR-1 | 38/59 | 27/59 | 27/59 |
| 1/2D CT-105–1/10D P3HR-1 | 14/46 | 13/46 | 13/46 |
| 1/2D CT-105–1/40D P3HR-1 | 8/44 | 5/44 | 5/44 |
| 1/10D CT-105 | 0/12 | 0/12 | 0/12 |
| 1/10D CT-105–1/2D P3HR-1 | 15/35 | 13/35 | 13/35 |
| 1/10D CT-105–1/10D P3HR-1 | 10/43 | 8/43 | 8/43 |
| Totals | | | |
| 1/2D CT-105 | 0/43 (0)[e] | 0/43 (0) | 0/43 |
| 1/2D CT-105–1/2D P3HR-1 | 57/96 (59) | 37/96 (39) | 37/96 |
| 1/2D CT-105–1/10D P3HR-1 | 17/75 (23) | 16/75 (21) | 16/75 |
| 1/2D CT-105–1/40D P3HR-1 | 0/52 (0) | 0/52 (0) | 0/52 |
| 1/10D CT-105 | 0/29 (0) | 0/29 (0) | 0/29 |
| 1/10D CT-105–1/2D P3HR-1 | 29/54 (53) | 20/54 (37) | 20/54 |
| 1/10D CT-105–1/10D P3HR-1 | 18/60 (30) | 15/60 (25) | 15/60 |
| 1/10D CT-105–1/40D P3HR-1 | 0/16 (0) | 0/16 (0) | 0/16 |

[a] 1/2D, 1/2 dilution; 1/10D, 1/10 dilution

[b] An LCL was considered positive if it amplified a PCR fragment across the P3HR-1 deletion.

[c] An LCL was considered positive if it amplified a the mutant PCR fragment across the EBNA-3C *Spe*I site.

[d] An LCL was considered positive if it amplified type 1 EBNA-3C.

[e] Numbers in parentheses represent the percentage of positive LCLs in the two experiments except for 1/10D CT-105–1/40D P3HR-1, values for which reflect only the numbers from experiment 1.

PCR, Southern hybridization, and immunoblot analysis of four representative MT EBNA-3C virus-infected progeny LCLs (E3CT-105.28A, E3CT-105.119A, E3CT-105.28B, and E3CT-105.82B) demonstrated their similarity to the MT EBNA-3C virus-infected parental E3CT-105 LCL. PCR with EBNA-3C primers amplified both type 1 and type 2 EBNA-3C in each of the four progeny MT EBNA-3C virus-infected LCLs (Fig. 8A, lanes 5 to 8). *Xba*I digestion of EBNA-3C *Spe*I PCR-amplified DNA from each progeny LCL resulted in the generation of two smaller fragments (Fig. 8C, lanes 5 to 8), demonstrating the presence of the *Xba*I/stop codon mutation in the EBNA-3C *Spe*I restriction site in each progeny LCL. PCR with the P3HR-Del primers amplified a 256-bp fragment in each of the MT EBNA-3C virus-infected progeny LCLs, indicating the presence of coinfecting parental P3HR-1 virus (Fig. 8B, lanes 5 to 8). PCR analysis with the type-specific EBNA-3A and -3B primers amplified both type 1 and 2 EBNA-3A and -3B in the progeny LCLs E3CT-105.28B and E3CT-105.119A (Fig. 5, lanes 5 and 6) as well as the parental E3CT-105 LCL (lane 4). Southern hybridization analysis of genomic DNA from the

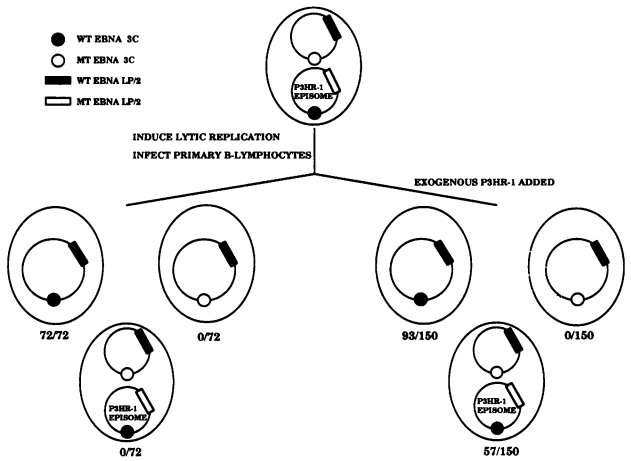


FIG. 7. Summary of the results of passage of an MT EBNA-3C recombinant EBV following induction of lytic infection in an LCL (E3CT-105) coinfected with the MT EBNA-3C and parental P3HR-1 EBV. Reactivated virus was used to infect primary B lymphocytes in the presence or absence of exogenously added parental P3HR-1 EBV to evaluate the dependence of the MT EBNA-3C virus on parental P3HR-1 virus to transform B lymphocytes.

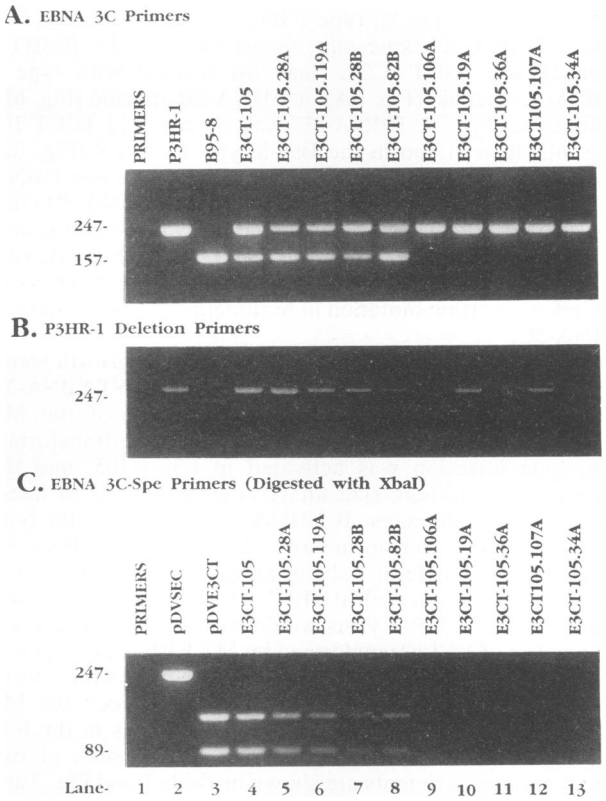


FIG. 8. PCR analysis of progeny LCLs infected with MT EBNA-3C recombinant EBV derived from E3CT-105, demonstrating the presence of the EBNA-3C mutation and coinfecting P3HR-1 virus. (A) EBNA-3C primers which distinguish between type 1 and type 2 EBNA-3C demonstrated the presence of both genes in the progeny MT EBNA-3C virus-infected LCLs. (B) Analysis with the P3HR-1 deletion primers demonstrated coinfection of the MT EBNA-3C virus-infected LCLs with P3HR-1 virus. (C) EBNA 3C *Spe*I primers amplify across the EBNA-3C *Spe*I site and *Xba*I cleaved at the new *Xba*I site. PCR analysis of the parental MT EBNA-3C virus-infected E3CT-105 LCL is shown in lane 4, and analyses of four progeny MT EBNA-3C virus-infected LCLs are shown in lanes 5 to 8. PCR analyses of five progeny type 2 EBNA-3C LCLs (generated by secondary recombination; see text) are shown in lanes 9 to 13. Size markers (base pairs) of *Rsa*I fragments of $\phi$X174 are shown on the left.

progeny LCLs E3CT-105.28B, E3CT-105.82B, and E3CT-105.119 (Fig. 4B, lanes 5 to 7) demonstrated the same mutant type 1 1.1- and 5.8-kb fragments and type 2 4.3-kb fragment as in the parental E3CT-105 LCL (Fig. 4B, lane 4) when the DNA was digested with *Xba*I and *Sal*I and hybridized with a radiolabeled EBNA-3C cDNA probe. Immunoblot analysis of the progeny E3CT-105.132 LCL with the type 1 EBNA-3C- and EBNA-3B-specific antibodies demonstrated that type 1 EBNA-3C was not expressed (Fig. 6A, lane 4) but type 1 EBNA-3B was expressed (Fig. 6, lane 4). Five progeny LCLs infected with virus resulting from secondary recombination between the MT EBNA-3C and parental P3HR-1 genomes amplified only type 2 EBNA-3C (Fig. 8A, lanes 9 to 13), indicating the loss of the type 1 EBNA-3C gene. Consistent with this loss is the absence of any PCR-amplified fragments when the DNA from these progeny LCLs were amplified with the type 1-specific EBNA-3C *Spe*I primers (Fig. 8C, lanes 9 to 13). Only two (E3CT-105.19A and E3CT-105.107A) of the five progeny LCLs were coinfected with parental P3HR-1 (Fig. 8B, lanes 10 and 12), indicating that secondary recombination which results in pairing a WT EBNA-LP/2 with WT type 2 EBNA-3C on a single EBV genome results in a fully transforming virus.

**One secondary recombinant virus in which mutant EBNA-3C has been replaced by WT EBNA-3C is able to transform primary B lymphocytes.** One progeny LCL infected with virus from E3CT-105 was of interest because it was infected with a single EBV genome produced by WT type 2 EBNA-3C replacement of the MT type 1 EBNA-3C gene in the doubly recombinant MT EBNA-3C genome as a consequence of recombination between the MT EBNA-3C and the parental P3HR-1 genome. PCR analysis of this LCL with the type-specific EBNA-3A, -3B, and -3C primers demonstrated a single EBV genome with type 1 EBNA-3A and type 2 EBNA-3B and -3C (Fig. 5, lane 7). These results are consistent with the MT EBNA-3C genome, which contains WT EBNA-LP/2, recombining with the P3HR-1 5′ to the EBNA-3B and 3′ to EBNA-3C, replacing the MT type 1 EBNA-3C with WT type 2 EBNA-3C and restoring the transforming ability of the MT EBNA-3C EBV genome. These results also indicate that the type 1 EBV DNA in E3CT-105 5′ to EBNA-3B and including the EBNA-3A gene is WT in transforming capacity. Thus, the defect is 3′ to the EBNA-3A gene. Since the EBNA-3B gene is not required for B-lymphocyte growth transformation (66), these data confirm that the EBNA-3C mutation is the basis for the MT E3CT-105 genome being unable to initiate B-lymphocyte growth transformation.

**Recovery of MT EBNA-3A recombinant EBV requires P3HR-1 coinfection.** Insertion of a stop codon into the EBNA 3A ORF decreased the frequency of recovering LCLs infected with a type 1 EBNA-3A recombinant virus. The frequency of recovering LCLs infected with a WT type 1 EBNA-3A recombinant was 10 to 15% as previously reported (65). These previous experiments were done in parallel with the first four experiments (Table 2) and serve as a control for the frequency of recovering MT type 1 EBNA-3A

TABLE 2. MT EBNA-3A recombination frequency

| Expt | No. of recombinant EBNA-3A LCLs/total no. of LCLS | |
|---|---|---|
| | MT type 1 EBNA-3A | WT type 1 EBNA-3A[a] |
| 1 | 2/69 | 10/107 |
| 2 | 1/49 | 3/39 |
| 3 | 0/74 | 9/68 |
| 4 | 2/84 | 11/91 |
| 5 | 0/91, 0/93[b] | 4/48 |
| 6 | 2/78, 2/89 | 4/64 |
| Total no. | 9/627 | 41/417 |
| Total % | 1.4 | 10 |
| Total % P3HR-1 coinfected | 100 | 80 |

[a] The results in experiments 1 to 4 were taken from a previous study which was performed in parallel with this study and served as a baseline control for mutant EBNA-3A analysis (65).

[b] In experiments 5 and 6, two different mutant EBNA-3A cosmid constructs were used.

recombinant virus. In a similar study with an EBNA-3B stop codon mutation, the frequencies of recovering LCLs infected with WT (12%) or MT (10%) type 1 EBNA-3B recombinant virus were nearly identical (66). Further, 50% of the MT or WT recombinant virus-infected LCLs were not coinfected with parental P3HR-1 and therefore had no WT EBNA-3B provided in *trans*. In contrast to the expected 10% frequency of obtaining EBNA-3A stop codon (MT EBNA-3A) recombinant virus-infected LCLs, only 1.4% (9 of 627) of the LCLs established by infection with virus following transfection P3HR-1 cells with EBNA-3A stop codon DNA were infected with an MT EBNA-3A recombinant virus. All nine LCLs contained both type 1 and type 2 EBNA-3A by PCR after expansion to $2 \times 10^5$ cells (data not shown). Of the nine LCLs, eight lost the MT EBNA-3A virus with initial LCL growth in vitro; the cells were subjected to 2 to 4 weeks of growth following identification of the mutant genomes. By expansion to $2 \times 10^6$ cells, the MT type 1 EBNA-3A gene was no longer detected with use of either the type 1 EBNA-3A or EBNA-3A *Spe*I site-specific primer. The ninth LCL (E3AT-27) was PCR positive for both WT type 2 and MT type 1 EBNA-3A but did not contain coinfecting P3HR-1, as assayed by PCR (data not shown) and Southern blot (Fig. 3, lane 4) analysis. The presence of both a WT type 2 and an MT type 1 EBNA-3A gene in an LCL without parental P3HR-1 is likely due to partially homologous or nonhomologous recombination between the transfected MT type 1 EBNA-3A *Sal*I-C DNA and the type 2 P3HR-1 genome, resulting in a single genome with both EBNA-3A genes. This type of recombination occurred with a low frequency in previous experiments (65, 66). Southern hybridization analysis of E3AT-27 with an EBNA-3A cDNA probe confirmed that the recombination was likely to be partially homologous (Fig. 9). Digestion of type 1 EBV DNA with *Bcl*I results in a 17.2-kb (B95-8; lane 2) fragment when probed with EBNA-3A cDNA, while type 2 EBV DNA has an additional *Bcl*I site, leaving most of the EBNA-3A DNA hybridizing to a 3.9-kb fragment (P3HR-1; lane 1). E3AT-27 has the 3.9-kb fragment expected from the P3HR-1 EBNA-3A DNA and an anomalous DNA fragment larger than that expected from the MT type 1 EBNA-3A DNA. The larger than expected fragment is consistent with homologous recombination at the 5′ end and nonhomologous recombination in the region encoding the type 1 EBNA-3A

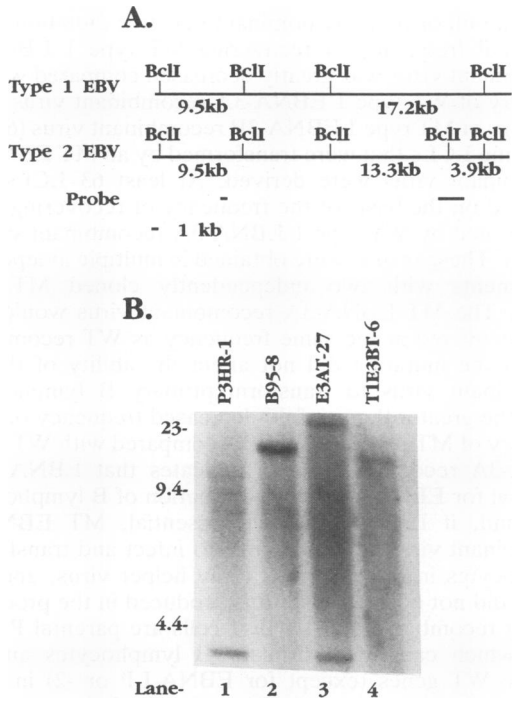


FIG. 9. Southern blot analysis of DNA from an LCL (E3AT-27) infected with MT EBNA-3A recombinant EBV. DNA was digested with restriction endonuclease *Bcl*I and hybridized with a $^{32}$P-radiolabeled EBNA-3A cDNA consisting of EBV positions 67649 to 67535 (from the splice site in *Bam*HI-U) and 92238 to 95234 (EBNA-3A ORF) (5). (A) Schematic of the provenance of the type 1 and type 2 EBNA-3A genes showing the *Bcl*I sites and fragments. Type 2 EBV (P3HR-1) has an additional *Bcl*I site (position 93135) in the EBNA 3A ORF, resulting in a prominent 3.9-kb fragment when hybridized with the EBNA-3A cDNA probe. In contrast, type 1 EBV (B95-8) lacks the EBNA-3A *Bcl*I site and has a prominent 17.2-kb *Bcl*I fragment. (B) Southern hybridization results for P3HR-1 (lane 1), B95-8 (lane 2), E3AT-27 (lane 3), and E3BT-6 (lane 4). E3AT-27 was the only LCL to grow out and maintain an MT EBNA-3A gene. E3BT-6 is a LCL infected with an recombinant EBV which has WT type 1 EBNA-3A and MT type 1 EBNA-3B genes. MT EBNA-3B virus-infected E3BT-6 is the positive control for a WT type 1 EBNA-3A recombinant LCL. The sizes (kilobases) of *Hin*dIII fragments of lambda DNA are shown on the left.

gene 5′ to the type 2 *Bcl*I site at 93135. Such a recombination pattern would result in the presence of both type 1 and 2 EBNA-3A, the presence of a normal type 2 3.9-kb *Bcl*I fragment, and the presence of a larger than normal 17.2-kb fragment due to the additional sequence from the type 2 EBNA-3A gene region.

## DISCUSSION

These studies demonstrate that EBNA-3A and -3C are essential for EBV growth transformation of primary B lymphocytes in vitro. This conclusion is based on a molecular genetic analysis using mutant recombinant EBVs with a stop codon inserted after codon 302 for EBNA-3A or codon 365 for EBNA-3C. Despite the likely translation of a third of the EBNA-3A or -3C ORF and the potential for downstream translational reinitiation (15, 32), no cross-reactive proteins were detected and both mutations proved to be null mutations.

Three lines of evidence indicate that the EBNA-3A muta-

tion is a null or partially dominant negative mutation. First, the initial frequency of recovering MT type 1 EBNA-3A recombinant virus was greatly decreased compared with the recovery of WT type 1 EBNA-3A recombinant virus or the recovery of MT type 1 EBNA-3B recombinant virus (65, 66). Only nine LCLs that were transformed by an MT EBNA-3A recombinant virus were derived. At least 63 LCLs were expected on the basis of the frequency of recovering LCLs transformed by WT type 1 EBNA-3A recombinant virus in parallel. These results were obtained in multiple independent experiments with two independently cloned MT EBV DNAs. The MT EBNA-3A recombinant virus would have been recovered at the same frequency as WT recombinant virus if the mutation did not affect the ability of the MT recombinant virus to transform primary B lymphocytes. Thus, the greater than sixfold-decreased frequency of initial recovery of MT type 1 EBNA-3A compared with WT type 1 EBNA-3A recombinant virus indicates that EBNA-3A is essential for EBV growth transformation of B lymphocytes.

Second, if EBNA-3A is not essential, MT EBNA-3A recombinant virus should be able to infect and transform B lymphocytes in the absence of any helper virus, and such events did not occur. Most virus produced in the process of making recombinants in P3HR-1 cells are parental P3HR-1 EBV which can infect primary B lymphocytes and can provide WT genes (except for EBNA-LP or -2) in *trans*. LCLs that were coinfected with an MT EBNA-3A recombinant and parental P3HR-1 EBV were initially obtained. This was a fortunate positive outcome indicating that MT EBNA-3A EBV recombinant virus was created. Infection of primary B lymphocytes with virus stocks from parallel P3HR-1 transfections which contained WT or MT type 1 EBNA-3B recombinant virus resulted in LCLs infected with WT or MT type 1 EBNA-3B recombinant virus, and in each case, half of the recombinant virus-infected LCLs were initially coinfected with parental P3HR-1 and half were transformed by the MT recombinant virus alone (66). In contrast, a null mutation in an essential gene is expected to be recovered only if the WT gene is provided in *trans*. Since about half of the MT EBNA-3A-infected primary B lymphocytes are expected to be initially coinfected with parental P3HR-1 EBV, which would provide WT EBNA-3A in *trans*, only a twofold reduction is expected from a null mutation in an essential gene, and 32 coinfected LCLs should have resulted. Thus, the observed sixfold (nine coinfected LCLs) versus expected twofold (32 expected coinfected LCLs) overall reduction is most compatible with the hypothesis that the EBNA-3A linker insertion after codon 302 results in a partial dominant negative effect (23), inhibiting the ability of WT EBNA-3A to *trans* complement the EBNA-3A mutation.

Third, the MT type 1 EBNA-3A genome not only was recovered less frequently than expected and always with coinfecting P3HR-1 EBV but also was quickly lost as the cultures were expanded. Eight of nine LCLs which initially contained both MT type 1 and WT type 2 EBNA-3A after expansion to $2 \times 10^5$ cells contained no detectable MT type 1 EBNA-3A recombinant virus following expansion to $1 \times 2 \times 10^6$ cells. The ninth LCL had both MT and WT EBNA-3A on a single genome produced by an anomalous recombination. This latter recombination is likely to result in nonexpression of the MT EBNA-3A, since the WT EBNA-3A appears to be in the normal EBNA-3A context and EBNA-3A is expressed from a distant promoter as part of a long, multiply spliced transcript. The rapid loss of the EBNA-3A mutation in the MT EBNA-3A virus-infected LCLs contrasts with the stable maintenance of the EBNA-3B mutation in MT EBNA-3B virus-infected LCLs coinfected with parental P3HR-1 virus (66). As the MT EBNA-3B virus-infected LCLs were expanded, the P3HR-1 EBV carrying WT EBNA-3B was frequently lost, since the coinfecting P3HR-1 genomes were not required to maintain growth transformation.

The rapid loss of the MT EBNA-3A recombinant genomes from the initially coinfected LCLs must involve not only a specific selection against cells infected with the mutant recombinant genomes but also a secondary recombination between the initially coinfecting parental P3HR-1 EBV genome which is deleted for EBNA-LP/2 and WT for type 2 EBNA-3A and the double recombinant genome which is WT for EBNA-LP/2 and MT for type 1 EBNA-3A, resulting in the generation of a transformation-competent WT EBNA-LP/2 and WT type 2 EBNA-3A EBV genome. Such a secondary recombination event could take place during S-phase replication of the two initial episomes in latently infected cells or could occur in the rare cell permissive for lytic virus replication. In the latter circumstance, the secondary EBV recombinant would infect an LCL already harboring two EBV episomes and enable the segregation of progeny cells infected with only the secondary recombinant episome. Whichever mechanism is operative, the inability to maintain the MT type 1 EBNA-3A recombinant virus is further evidence in support of the requirement for WT EBNA-3A for B-lymphocyte growth transformation and of the probable selection against LCLs coinfected with the MT EBNA-3A recombinant and P3HR-1 virus as the result of a partial dominant negative effect of the EBNA-3A mutation.

The truncated type 1 EBNA-3A protein could interfere with normal EBNA-3A function by acting as a competitive inhibitor of the interaction of the WT type 2 EBNA-3A with an important effector, resulting in an overall loss of function (23). Under such conditions, the expected frequency of recovering mutant virus would be decreased even in the presence of the WT protein provided in *trans*.

Similar evidence indicates that the EBNA-3C mutation is a null mutation in an essential gene for primary B-lymphocyte growth transformation. MT type 1 EBNA-3C recombinant virus was recovered less frequently than was WT virus. In several experiments using two different MT EBNA-3C cosmids, only rarely was MT type 1 EBNA-3C recombinant virus recovered. However, because of the low frequency of recovering even WT type 1 EBNA-3C recombinant virus, a meaningful comparison with recovery of MT type 1 EBNA-3C recombinant virus cannot be made. All five LCLs which were recovered contained both MT type 1 and WT type 2 EBNA-3C. Four of the five were coinfected with parental P3HR-1 EBV. In three of the four coinfected LCLs, the MT type 1 EBNA-3C recombinant genome was slowly lost as the cultures were expanded. The loss of the MT EBNA-3C recombinant virus was slower than that observed with MT EBNA-3A recombinant virus, as evidenced by the ability to expand the MT EBNA-3C virus-infected LCLs beyond the $1 \times 10^6$ to $2 \times 10^6$-cell stage and the fact that one LCL (E3CT-105) stably maintained both the MT EBNA-3C and parental P3HR-1 EBV genomes over 1 year in continuous passage. The decreased ability to recover an LCL infected with an MT EBNA-3C recombinant virus alone and the inability to maintain the MT type 1 EBNA-3C recombinant virus in most LCLs indicate that EBNA-3C, like EBNA-3A, is essential for EBV growth transformation of B lymphocytes. The slower loss and retention of one MT

EBNA-3C recombinant genome is most compatible with a null rather than dominant negative effect of the mutation.

The ability to recover and maintain the MT type 1 EBNA-3C recombinant virus was strictly dependent on parental P3HR-1 EBV coinfection. In LCL E3CT-105, in which the coinfecting genomes were stably maintained, the long-term maintenance of parental P3HR-1 EBV contrasts with the gradual loss of parental P3HR-1 EBV genomes from WT type 1 EBNA-3 and MT type 1 EBNA-3B recombinant-coinfected LCLs. In these coinfected LCLs, the parental P3HR-1 is lost over time as the LCLs are maintained in culture (65, 66). The stable maintenance of parental P3HR-1 EBV in E3CT-105 is consistent with a requirement for parental P3HR-1 EBV to provide WT EBNA-3C in *trans*. An absolute dependence on parental P3HR-1 EBV was further demonstrated by serial passage of the MT EBNA-3C recombinant virus to new primary B lymphocytes in the presence of exogenous parental P3HR-1 EBV. MT EBNA-3C virus transformed primary B lymphocytes only when WT EBNA-3C was provided in *trans* by P3HR-1 EBV coinfection. In contrast, progeny LCLs singly infected with WT type 1 EBNA-3A or MT EBNA-3B recombinant virus were easily established (65, 66). The frequency of recovery of LCLs infected with an MT EBNA-3C recombinant virus was strictly dependent on the dilution of exogenous coinfecting parental P3HR-1 virus. These results are the most conclusive evidence that EBNA-3C is essential for growth transformation of B lymphocytes and that the mutation inactivates the essential EBNA-3C function. This conclusion is further supported by the serendipitous derivation of a secondary recombinant virus in which the MT EBNA-3C gene was replaced by WT EBNA-3C gene, resulting in a recombinant virus which was able to transform primary B lymphocytes.

EBNA 3A, -3B, and -3C are hydrophilic, charged, nuclear proteins with similar intranuclear distributions and similar sizes of approximately 1,000 amino acids (22, 26, 28, 45–47, 53, 58). Each has a potential hydrophobic leucine or isoleucine intermolecular interactive domain near amino acid 270 (2, 6), and each has peptide repeat domain near amino acid 700. The proximity of the EBNA-3 genes in the EBV genome, their similar exon structures, and the protein similarities are compatible with these genes being similar in evolution or function. The only known function for this gene family is the effect of EBNA-3C in upregulating CD21 protein and mRNA in EBV-negative lymphoma cells (69). EBNA-3C may also increase LMP-1 expression in the EBV-positive Burkitt lymphoma line (3) Raji, which contains an EBV deleted for the EBNA-3C coding region and therefore does not express EBNA 3C (48). EBNA-3A could interact with EBNA-3C or with a parallel set of effectors. The potential hydrophobic interactive domains are included in the translated part of the EBNA-3A or -3C mutant protein, and the mutant EBNA-3A polypeptide may be sufficiently stable to block normal interactions of this domain. Studies are now under way to directly determine whether the truncated EBNA-3A protein has a partial dominant negative effect on WT EBNA-3A function. If so, this finding may be a link to an important EBNA-3A effector.

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